



# Antioxidative responses of three oak species under ozone and water stress conditions

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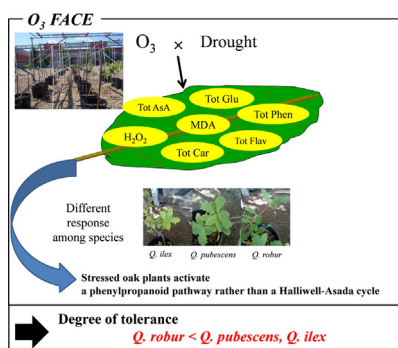
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## HIGHLIGHTS

- Detoxification mechanisms at the basis of the high plasticity of oak species
- Ozone and drought had antagonistic effects on biochemical traits.
- Intrinsic physiological and biochemical mechanisms contribute to stress tolerance.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Plants are frequently exposed to adverse environmental conditions such as drought and ozone ( $O_3$ ). Under these conditions, plants can survive due to their ability to adjust their metabolism. The aim of the present study was to compare the detoxification mechanisms of three oak species showing different  $O_3$  sensitivity and water use strategy. Two-year-old seedlings of *Quercus ilex*, *Q. pubescens* and *Q. robur* were grown under the combination of three levels of  $O_3$  (1.0, 1.2 and 1.4 times the ambient  $O_3$  concentration) and three levels of water availability (on average 100, 80 and 42% of field capacity i.e. well-watered, moderate drought and severe drought, respectively) in an  $O_3$  Free Air Controlled Exposure facility. Ozone and drought induced the accumulation of reactive oxygen species (ROS) and this phenomenon was species-specific. Sometimes, ROS accumulation was not associated with membrane injury suggesting that several antioxidative defence mechanisms inhibited or alleviated the oxidative damage. Both  $O_3$  and drought increased total carotenoids that were able to prevent the peroxidation action by free radicals in *Q. ilex*, as confirmed by unchanged malondialdehyde by-product values. The concomitant decrease of total flavonoids may be related to the consumption of these compounds by the cell to inhibit the accumulation of hydrogen peroxide. Unchanged total phenols confirmed that *Q. ilex* has a superior ability to counteract oxidative conditions. Similar responses were found in *Q. pubescens*, although the negative impact of both factors was less efficiently faced than in the sympatric *Q. ilex*. In *Q. robur*, high  $O_3$  concentrations and severe drought induced a partial rearrangement of the phenylpropanoid pathways. These antioxidative mechanisms were not able to protect the cell structure (as confirmed by ROS accumulation) suggesting that *Q. robur* showed a lower degree of tolerance than the other two species.

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## 1. Introduction

Evidence for changing climate, associated with higher atmospheric concentrations of greenhouse gases, continues to increase. The years 2014 and 2015 are currently considered the warmest years in Europe since instrumental records began, i.e. >1.1 °C warmer than the pre-industrial level (EEA, 2017). For these years, the exceptional heat covered the whole summertime with mean precipitation (June to August) significantly decreased by up to 20 mm per decade. The series of summer heatwaves affecting Europe since 2003 has also contributed to several intense tropospheric ozone (O<sub>3</sub>) episodes. In 2015, 18 of the 28 states of the European Union (EU) and four other European countries outside the EU registered concentrations above the EU O<sub>3</sub> target value for the protection of human health (EEA, 2017).

Due to favorable meteorological conditions, both drought and O<sub>3</sub> are very likely to occur simultaneously. As a general rule, seasonal drought is typically associated with high insolation, and such conditions are conducive to the photo-oxidative formation of high O<sub>3</sub> levels (Butkovic et al., 1990). In addition, a rise in temperature significantly increases the emission rates of most biogenic volatile organic compounds that can contribute to O<sub>3</sub> production (Avery, 2006). Under drought, plants usually suffer from the impairment of many physiological and biochemical processes, such as (i) alteration of photosynthetic performance, (ii) cell dehydration, (iii) high production of reaction oxygen species (ROS) and, finally, (iv) early senescence and/or leaf necrosis (Chaves et al., 2003). Similar effects have also been attributed to O<sub>3</sub> (Cotrozzi et al., 2017a; Jolivet et al., 2016). A combination of drought and O<sub>3</sub> can induce responses considerably different from those observed when each stressor is applied independently (Bohler et al., 2015). Interestingly, the effects of drought and O<sub>3</sub> can be antagonistic, so that a simultaneous occurrence may be partially beneficial to plants. The most common combined response, in fact, is that drought mitigates the negative effects of O<sub>3</sub>, basically by closing stomata and thus reducing O<sub>3</sub> uptake into the plant (Pollastrini et al., 2013; Gao et al., 2017). However, other results suggest that drought can exacerbate O<sub>3</sub> damage: Alonso et al. (2014) reported that the combination of both stressors caused further decreases in accumulated aboveground biomass in two subspecies of *Quercus ilex*. It appears that the combination of drought and O<sub>3</sub> is highly dependent on (i) the severity and length of occurrence of both stress factors and (ii) the balance between stomatal O<sub>3</sub> uptake (i.e., Phytotoxic O<sub>3</sub> Dose, POD) and detoxification capacity of foliar cells (Dizengremel et al., 2013; Bohler et al., 2015).

Some studies have investigated the effects of combined drought and O<sub>3</sub> exposure on plant metabolism, especially in trees (see also Pollastrini et al., 2013; Cotrozzi et al., 2016; Yuan et al., 2016; Gao et al., 2017; Cotrozzi et al., 2017b). However, none of them investigated antioxidant molecules and/or physiological mechanisms. Whereas O<sub>3</sub> itself induces production of ROS and leads to a strong ROS accumulation, physiological responses to drought mostly use ROS as internally produced signalling molecules (Reddy et al., 2004), and severe drought may lead to photo-oxidative stress (Czarnocka and Karpiński, 2018). Consequently, accumulation of ROS is likely to be considerably higher during O<sub>3</sub> stress, and more closely located to chloroplasts under drought stress. Recently, Cotrozzi et al. (2017b) documented that hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and superoxide anion (O<sub>2</sub><sup>-</sup>) were directly involved in the O<sub>3</sub>-oxidative burst induced by an intense episode of O<sub>3</sub> exposure (200 ppb for 5 h) in three-year-old *Q. ilex* saplings. By contrast, H<sub>2</sub>O<sub>2</sub> content did not change in plants subjected to drought (20% of the effective daily evapotranspiration, for 15 days). Such differences in ROS extent dynamics in relation to the stress factor suggested a complex network of events in signal transduction, involving other molecules (e.g., salicylic and jasmonic acid) and processes (e.g., proline biosynthesis).

Oaks (belonging to the genus *Quercus*) are widely distributed trees within the Mediterranean area and are able to cope with several environmental stressors due to the high plasticity of their phenotypic and physiological traits (Cotrozzi et al., 2016). Holm oak (*Q. ilex*) is likely

the most widely studied Mediterranean evergreen tree species and has been defined as “drought avoidant” (Bussotti et al., 2002) and “O<sub>3</sub>-tolerant” (Cotrozzi et al., 2018a; Hoshika et al., 2018). Downy oak (*Q. pubescens*) is a typical Mediterranean deciduous tree distributed in Southern Europe and has been defined as “drought-” (Curtu et al., 2011) and “O<sub>3</sub>-tolerant” (Cotrozzi et al., 2018a; Hoshika et al., 2018). Pedunculate oak (*Q. robur*) is one of the basic species in deciduous broadleaved forests of Europe and has been defined as “moderately drought-tolerant” (Vranckx et al., 2014) and “O<sub>3</sub>-sensitive” (Hoshika et al., 2018).

The aim of the present study was to assess the combined effects of drought and O<sub>3</sub> exposure on the antioxidant metabolism of three oak species showing different water use strategies and O<sub>3</sub> sensitivities, exposed for one growing season to three levels of water availability and three levels of O<sub>3</sub> in an O<sub>3</sub> Free Air Controlled Exposure (FACE) facility. Specifically, we asked the following questions: (i) How much ROS are induced by realistic O<sub>3</sub> and water stress levels? (ii) Which antioxidant mechanisms are activated in response to individual stresses and to the combination of the stressors at different intensities? (iii) Are metabolic responses markedly species-specific? (iv) Are antioxidative metabolism and stomatal uptake of O<sub>3</sub> correlated? We postulated a protective effect of drought against O<sub>3</sub> and that the interactive effects of both factors may depend on plant species. In particular, we hypothesized that the evergreen tree species (which usually inhabits limiting environments) will have a greater tolerance to drought and O<sub>3</sub> exposure than the deciduous ones (characterized by shorter leaf lifespan), due to its stronger need to protect its long-lived leaves from different environmental cues. In a previous work, Cotrozzi et al. (2016) demonstrated that *Q. ilex* was able to successfully cope with several stressors due to the high plasticity of morpho-anatomical, physiological and biochemical traits.

## 2. Materials and methods

### 2.1. Plant material and experimental design

At the beginning of autumn 2014, two-year-old saplings of *Q. ilex*, *Q. pubescens* and *Q. robur* were transferred from nearby nurseries to the O<sub>3</sub>-FACE facility of Sesto Fiorentino, Florence, Italy (43°48′59″N, 11°12′01″E, 55 m a.s.l.), where the experimental activities were conducted. The plants were established into 10-L pots containing peat: sand:nursery soil (1:1:1 in volume) and maintained under field conditions until the beginning of the treatment. Uniform-sized plants were selected and grown under the combination of three levels of O<sub>3</sub> (1.0, 1.2 and 1.4 times the ambient air concentration, denoted as AA, 1.2 × AA and 1.4 × AA, respectively) and three levels of water irrigation [100, 80 and 42% of field capacity on average, denoted as WW (well watered), MD (moderate drought) and SD (severe drought), respectively] from 1st June to 15th October 2015 (4.5 months). A detailed description of the O<sub>3</sub> exposure methodology is available in Paoletti et al. (2017). The maximum hourly ozone concentrations were 93 ppb in AA, 111 ppb in 1.2 × AA and 123 ppb in 1.4 × AA, respectively, throughout the experimental period. AOT40 (Accumulated exposure Over Threshold of 40 ppb) values during the experimental period were 17.8 ppm h, 29.7 ppm h and 40.3 ppm h in AA, 1.2 × AA and 1.4 × AA, respectively. Biomass results from this experiment were used for assessing O<sub>3</sub> risk in a previous paper (Hoshika et al., 2018), where further details on O<sub>3</sub> metrics are also available. The amount of irrigation was related to the soil field capacity, i.e. the maximum volume of water that was retained into the soil of the pots [volumetric soil water content was measured in the root layer by EC-5 soil moisture sensors equipped with an EM5b data logger, (Decagon Devices, Pullman, WA, USA), Hoshika et al. (2018)]. Three replicated plots (5 × 5 × 2 m) were assigned to each O<sub>3</sub> treatment, with three plants per each combination of species, O<sub>3</sub> level and water irrigation. At the end of the experiment, the first mature (fully expanded) top leaves of all three plants per plot (one leaf with 5th to 8th order per plant) in each O<sub>3</sub> × irrigation

treatment were gathered, divided into aliquots (obtained from each combination of species, O<sub>3</sub> level and water irrigation per plot), immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until biochemical analyses were done. Sampling was performed from 11:00 am to 1:00 pm.

## 2.2. Oxidative damage and H<sub>2</sub>O<sub>2</sub> content

Oxidative damage to membranes was estimated in terms of lipid peroxidation by determining the malondialdehyde (MDA) by-product accumulation, according to the method of Guidi et al. (2017). Samples (40 mg fresh weight, FW) were extracted with 1 mL of 0.1% (w/v) trichloroacetic acid. The determination was performed with a spectrophotometer (6505 UV-Vis, Jenway, UK) at 532 and 600 nm.

H<sub>2</sub>O<sub>2</sub> content was measured fluorometrically using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA), according to Cotrozzi et al. (2017b). Samples (30 mg FW) were extracted with 800  $\mu\text{L}$  of 20 mM potassium-phosphate (K-P) buffer (pH 6.5). The determination was performed with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, USA) at 530 and 590 nm (excitation and emission of resorufin fluorescence, respectively).

## 2.3. Pigments

Pigments were assessed according to Cotrozzi et al. (2017b), with minor modifications. Samples (30 mg FW) were homogenized in 1 mL of 100% HPLC-grade methanol and incubated overnight at  $4^{\circ}\text{C}$  in the dark. High Performance Liquid Chromatography (HPLC; P680 Pump, UVD170U UV-VIS detector, Dionex, Sunnyvale, CA, USA) separation was performed at room temperature with a reverse-phase Dionex column [Acclaim 120, C18, 5  $\mu\text{m}$  particle size, 4.6 mm internal diameter (i.d.) 150 mm length]. A detailed description of analytical conditions is available in Cotrozzi et al. (2017b).

## 2.4. Metabolites involved in the Halliwell-Asada cycle

Contents of ascorbate and glutathione were assessed according to Davey et al. (2003), with minor modifications. Samples (100 mg FW) were extracted in 1 mL of chilled extraction buffer [6% metaphosphoric acid in 65% HPO<sub>3</sub> (w/v), pH 1.5] containing 2 mM EDTA and 1% polyvinylpyrrolidone (w/w). The supernatant was divided in order to determine the reduced form of ascorbate and glutathione (i.e. AsA and GSH, respectively) and the total pool of each component (total ascorbate and total glutathione). To determine total pools, extracts were mixed with 200 mM dithiothreitol in 2 M Tris base to reach a pH between 6.0 and 6.8. HPLC separation was performed on a Prominence Shimadzu system (LC-20AT pump, SPD-M20A diode array detector, Shimadzu, Tokyo, Japan) at  $25^{\circ}\text{C}$  with a reverse-phase column (Kinetex EVO C18, 2.6  $\mu\text{m}$  spherical particle size, 4.6 mm i.d., 100 mm length).

## 2.5. Metabolites involved in the phenylpropanoid pathway

Contents of phenylpropanoids were assessed according to Cotrozzi et al. (2018b), with some modifications. Samples (30 mg FW) were homogenized in 500  $\mu\text{L}$  of 80% HPLC-grade methanol [in water (v/v)]. The supernatant was diluted five-fold with an aqueous solution of 0.2% formic acid. UHPLC-ESI-MS/MS analyses were performed on an Agilent 1290 Infinity II LC system coupled to a 6495 Triple Quadrupole mass spectrometer equipped with a Jet Stream electrospray (ESI) ionization source (Agilent Technologies, Santa Clara, CA, USA). The separation was achieved at  $35^{\circ}\text{C}$  using a reverse-phase Agilent column (Zorbax Eclipse Plus, C18, 1.8  $\mu\text{m}$  particle size, 2.1 mm i.d., 50 mm length). A detailed description of analytical conditions is available in Assumpção et al. (2018). In the present study, phenylpropanoid metabolites are

grouped and presented as total free phenolic acids (Tot Phen) and flavonoids (Tot flav) on the basis of their chemical structure.

## 2.6. Relationship of oxidative metabolism and stomatal ozone uptake (POD)

The O<sub>3</sub> dose during the experiment was calculated as the phytotoxic ozone dose (POD<sub>y</sub>) above an hourly stomatal uptake threshold of  $0\text{ nmol m}^{-2}\text{ s}^{-1}$  (POD<sub>0</sub>) determined for each oak species in our previous work (Hoshika et al. 2018), in order to assess relationships between the observed parameters of oxidative metabolism and stomatal O<sub>3</sub> uptake. POD<sub>0</sub> was calculated by considering species-specific stomatal responses to environmental stimuli according to the Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends (CLRTAP, 2017). The details were described in Hoshika et al. (2018).

## 2.7. Statistical analyses

Statistical analyses were performed with Microsoft Office Excel 2010 (Microsoft, Redmond, WA, USA) and JMP 11.0 (SAS Institute, Cary, NC, USA). The statistical unit was the single plot, i.e. all leaves from the three plants per species in each plot were merged in a single sample per O<sub>3</sub>  $\times$  irrigation replicate (N = 3 plots). Data for all biochemical parameters were tested with the Shapiro-Wilk W test for normality and with the Levene test for homogeneity of variance. All data were normally distributed and thus were analyzed by three-way ANOVA with O<sub>3</sub>, drought and species as fixed factors. Comparisons among means were determined by the Tukey HSD post-test. MDA data were analyzed using one-way ANOVA followed by the Tukey HSD post-test. To determine whether O<sub>3</sub> and drought exerted additive, synergistic or antagonistic impacts on the traits, the observed (Obs) effects were compared with the expected (Exp) additive ones for each oak species exposed to both stressors, according to Bansal et al. (2013). When the difference between Obs and Exp was positive and the lower 95% confidence limit was greater than zero, the impact from the combined stressors was classified as “synergistic”. When the difference between Obs and Exp was negative and the upper 95% confidence limit was less than zero, the impact from the combined impact was classified as “antagonistic”. When the 95% confidence interval crossed the zero line, the impact was classified as “additive”. The relationships between oxidative metabolism and POD<sub>0</sub> of the three oak species were tested by a simple linear regression analysis, and species differences in statistically significant regressions were evaluated by analysis of covariance (ANCOVA).

## 3. Results

### 3.1. Oxidative damage and H<sub>2</sub>O<sub>2</sub> content

For MDA, the interaction among O<sub>3</sub>, drought and species was not significant (Table 1, Fig. 1S). The effects of single factors and their interactions in all binary combinations (O<sub>3</sub>  $\times$  drought; O<sub>3</sub>  $\times$  species and drought  $\times$  species) were significant. Ozone *per se* had a significant impact on MDA only in the deciduous species. In *Q. pubescens*, O<sub>3</sub> markedly increased the values of MDA, without significant differences between the two higher O<sub>3</sub> concentrations (one-way ANOVA with O<sub>3</sub> as factor:  $P \leq 0.001$ ). Only moderate O<sub>3</sub> concentrations induced an accumulation of MDA in *Q. robur* (one-way ANOVA with O<sub>3</sub> as factor:  $P \leq 0.05$ ). Drought *per se* had a significant impact on MDA only in the deciduous species. In *Q. pubescens*, drought markedly increased MDA, without significant differences between reduced water availability (one-way ANOVA with drought as factor:  $P \leq 0.001$ ). Only MD induced an accumulation of MDA in *Q. robur* (one-way ANOVA with drought as factor:  $P \leq 0.05$ ). *Q. ilex* displayed MDA values about 2-fold higher than the other species (one-way ANOVA with plant species as factor:  $P \leq 0.001$ ) and no effects of O<sub>3</sub> and drought were observed.

**Table 1**

*P* values of three-way ANOVA of the effects of ozone ( $O_3$ ), drought and plant species on malondialdehyde (MDA), hydrogen peroxide ( $H_2O_2$ ), total carotenoids (Tot Car), total ascorbate (Tot AsA), total glutathione (Tot Glu), total phenolic acids (Tot Phen) and total flavonoids (Tot Flav). Asterisks show the significance of factors/interaction: \*\*\*  $P \leq 0.001$ , \*\*  $P \leq 0.01$ ; ns  $P > 0.05$ . d.f. represents the degrees of freedom.

Effects	d. f.	MDA	$H_2O_2$	Tot Car	Tot AsA	Tot GSH	Tot Phen	Tot Flav
$O_3$	2	***	***	***	***	*	***	***
Drought	2	***	***	**	**	ns	ns	ns
Plant species	2	***	***	***	***	***	***	***
$O_3 \times$ drought	4	**	***	***	ns	ns	***	***
$O_3 \times$ plant species	4	**	***	***	ns	ns	***	***
Drought $\times$ plant species	4	***	***	***	*	ns	***	***
$O_3 \times$ drought $\times$ plant species	8	ns	***	***	ns	ns	***	***

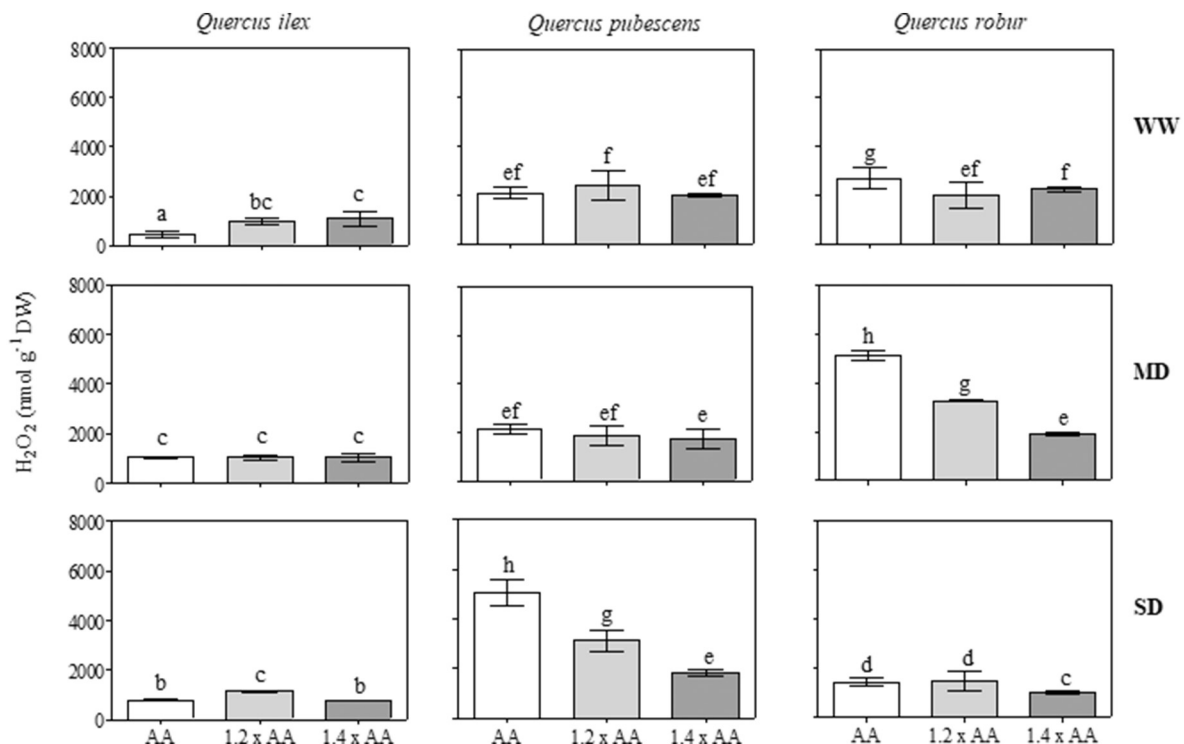
The effects of all combinations of  $O_3$ , drought and species were significant for  $H_2O_2$  (Table 1, Fig. 1).  $O_3$  *per se* increased the content of  $H_2O_2$  in *Q. ilex* WW plants, without significant differences between the two higher  $O_3$  concentrations (Fig. 1). Drought *per se* markedly increased  $H_2O_2$  in *Q. ilex* under AA conditions, with significant differences between reduced water availability (+127 and +74% under MD and SD conditions, compared to WW). *Q. pubescens* exhibited a SD-induced accumulation of  $H_2O_2$  (about 2-fold higher than WW). Similarly, *Q. robur* exhibited a MD-induced accumulation of  $H_2O_2$  (+89% compared to WW). Under AA-WW conditions, *Q. ilex* displayed  $H_2O_2$  values about 5-fold lower than the other species. Under combined conditions ( $O_3$  and drought),  $H_2O_2$  contents remained unaltered in *Q. ilex* exposed to increasing  $O_3$  levels, independently of the watering regimes, except for  $1.2 \times$  AA combined with SD (+44% in comparison to AA-SD).

Ozone and drought in combination had synergistic effects on  $H_2O_2$  in *Q. ilex*, except when the higher  $O_3$  concentrations and SD treatments were combined as in this case they acted antagonistically (Fig. 2A).

The two stressors generally had antagonistic effects on  $H_2O_2$  content also in deciduous species (Fig. 2A–C). Weak additive effects, however, were found in *Q. pubescens* when moderate levels of  $O_3$  were combined with SD, and in *Q. robur* exposed to the highest intensity of both stressors (Fig. 2B–C).

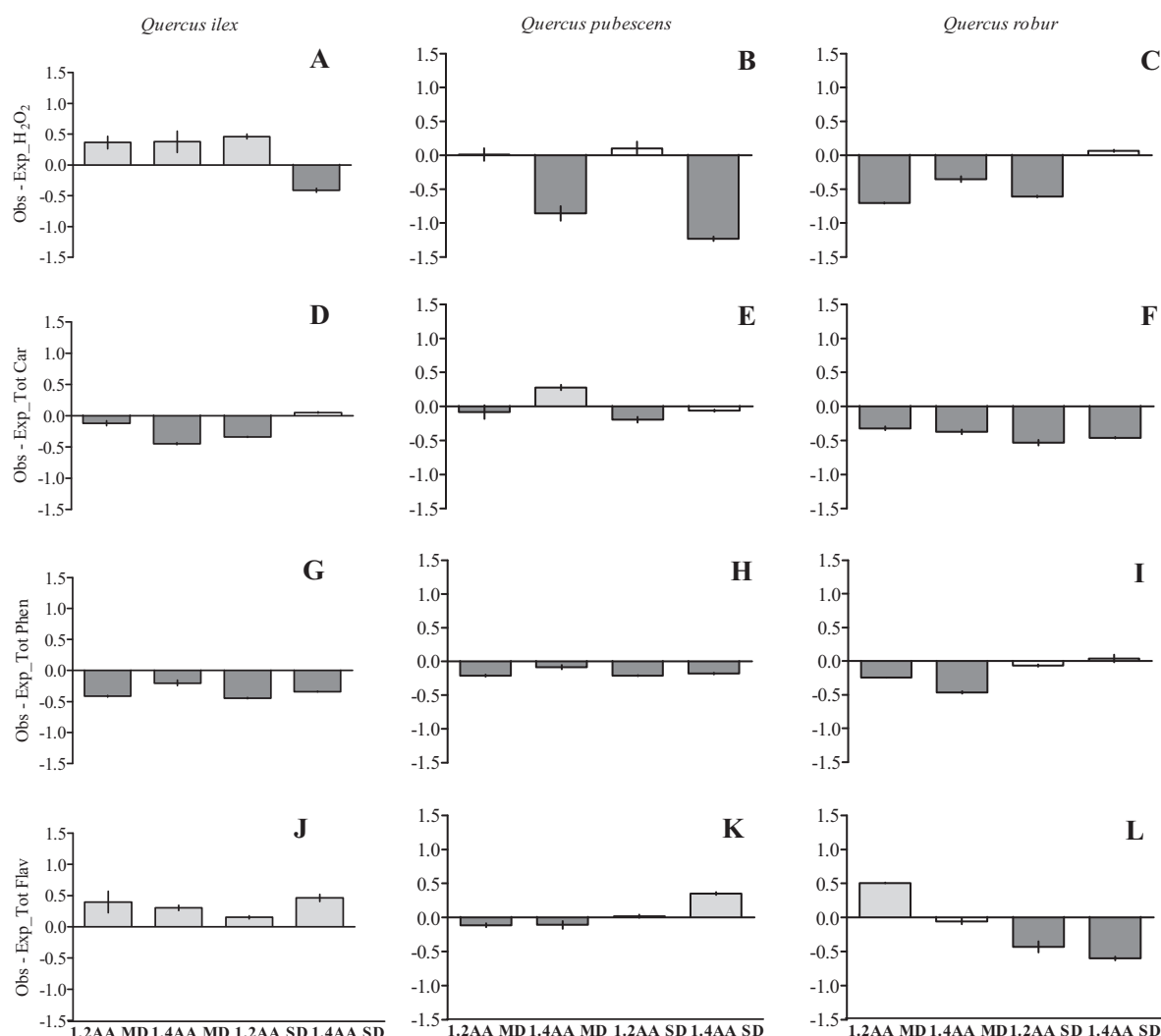
### 3.2. Total carotenoids

The effects of all combinations of  $O_3$ , drought and species on total carotenoids (Tot Car) were significant (Table 1, Fig. 3).  $O_3$  *per se* induced a slight accumulation of Tot Car in *Q. ilex* WW plants in comparison to AA ones, without significant differences between the higher  $O_3$  concentrations. High  $O_3$  concentrations decreased the content of Tot Car in *Q. pubescens* (–19% in comparison to AA conditions) and even more in *Q. robur*, with significant differences between increasing  $O_3$  levels (–28 and –38% in  $1.2 \times$  AA and  $1.4 \times$  AA, respectively). Drought *per se* also affected Tot Car in all species (except in *Q. ilex* under MD and in *Q. pubescens* under SD) in comparison to WW conditions. An SD-induced accumulation of Tot Car content occurred in *Q. ilex* (+45% compared to WW), while MD slightly decreased the levels of these metabolites in *Q. pubescens* (–18% in comparison to WW). Drought *per se* decreased Tot Car of *Q. robur*, with significant differences between drought regimes (–40 and –51% under MD and SD compared to WW). Under combined conditions ( $O_3$  and drought), Tot Car content was significantly affected in all species exposed to the higher  $O_3$  concentrations and subjected to reducing watering regimes in comparison to AA (except for *Q. ilex* MD plants). In particular,  $O_3$  induced a decrease of Tot Car in *Q. ilex* and *Q. pubescens* SD plants, with significant differences between increasing  $O_3$  levels (–23 and –82%, –26 and –12% in  $1.2 \times$  AA and  $1.4 \times$  AA, respectively). An opposite trend of Tot Car was observed in *Q. pubescens* MD plants in response to increasing  $O_3$  levels: –18 and +39% compared to AA ones (in  $1.2 \times$  AA and  $1.4 \times$  AA, respectively). An  $O_3$ -induced accumulation of Tot Car was also observed in *Q. robur* MD and SD plants, with



**Fig. 1.** Hydrogen peroxide [ $H_2O_2$ , nmol g<sup>-1</sup> dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air  $O_3$  exposure [applied for 4.5 months: ambient air (AA),  $1.2 \times$  and  $1.4 \times$  ambient  $O_3$  ( $1.2 \times$  AA and  $1.4 \times$  AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean ( $n = 3$ )  $\pm$  standard deviation. According to a three-way ANOVA with  $O_3$ , drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test,  $P \leq 0.05$ ).





**Fig. 2.** The combined impact of increasing ozone levels [applied for 4.5 months: 1.2  $\times$  and 1.4  $\times$  ambient air (1.2AA and 1.4AA)] and reducing water availability [moderate drought (MD) and severe drought (SD)] on hydrogen peroxide ( $H_2O_2$ , A–C), total carotenoids (Tot Car, D–F), total phenols (Tot Phen, G–I) and total flavonoids (Tot Flav, J–L) in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves. The bars represent the intensity-specific effect size difference (mean  $\pm$  95% confidence interval) between the observed (Obs) and expected (Exp) additive effects from the combination of the two stressors. The zero line represents the expected additive effects from combined stressors. When the means were greater than or less than the zero line, they were considered synergistic (gray bars) or antagonistic (dark gray bars; Bansal et al., 2013).

significant differences between increasing  $O_3$  levels (+27 and +81%, +157 and +55% in 1.2  $\times$  AA and 1.4  $\times$  AA, respectively).

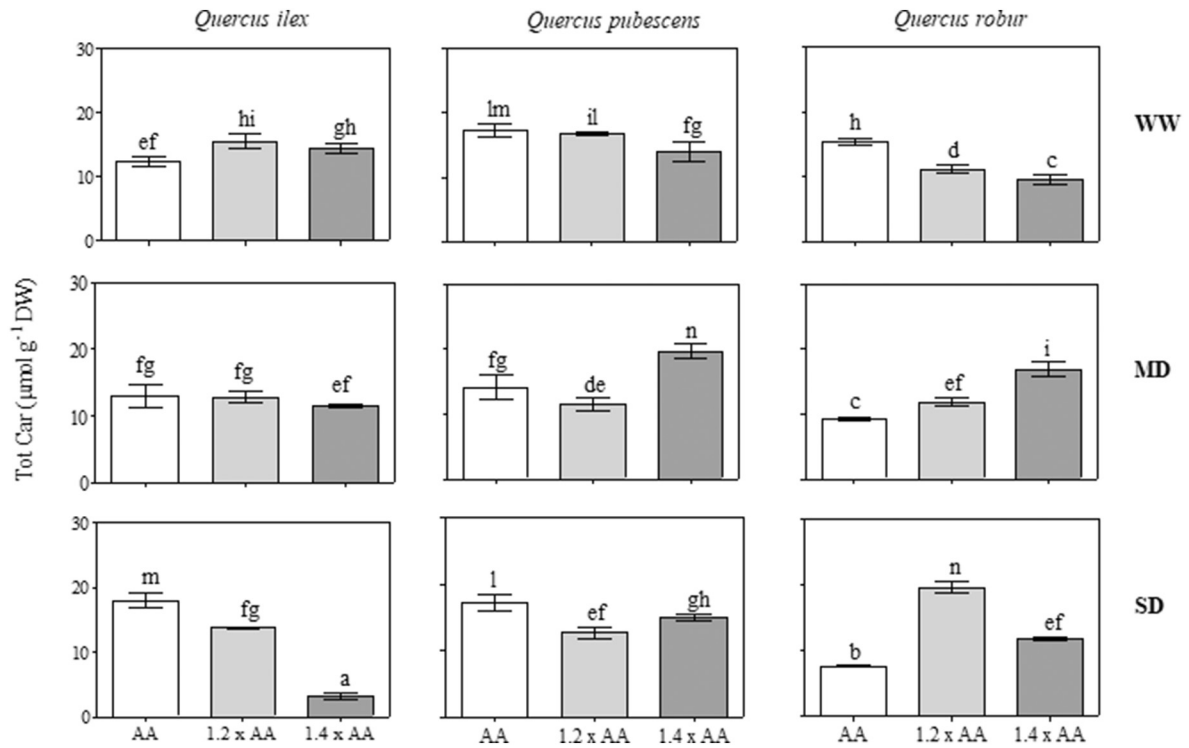
Ozone and drought in combination generally had antagonistic effects on Tot Car in all species (Fig. 2D–F). Additive effects were found in *Q. ilex* and in *Q. pubescens* when both stressors were of severe intensity. Synergistic effects were found only in *Q. pubescens* when high  $O_3$  concentrations and MD treatments were combined.

### 3.3. Metabolites involved in the Halliwell-Asada cycle and in the phenylpropanoid pathway

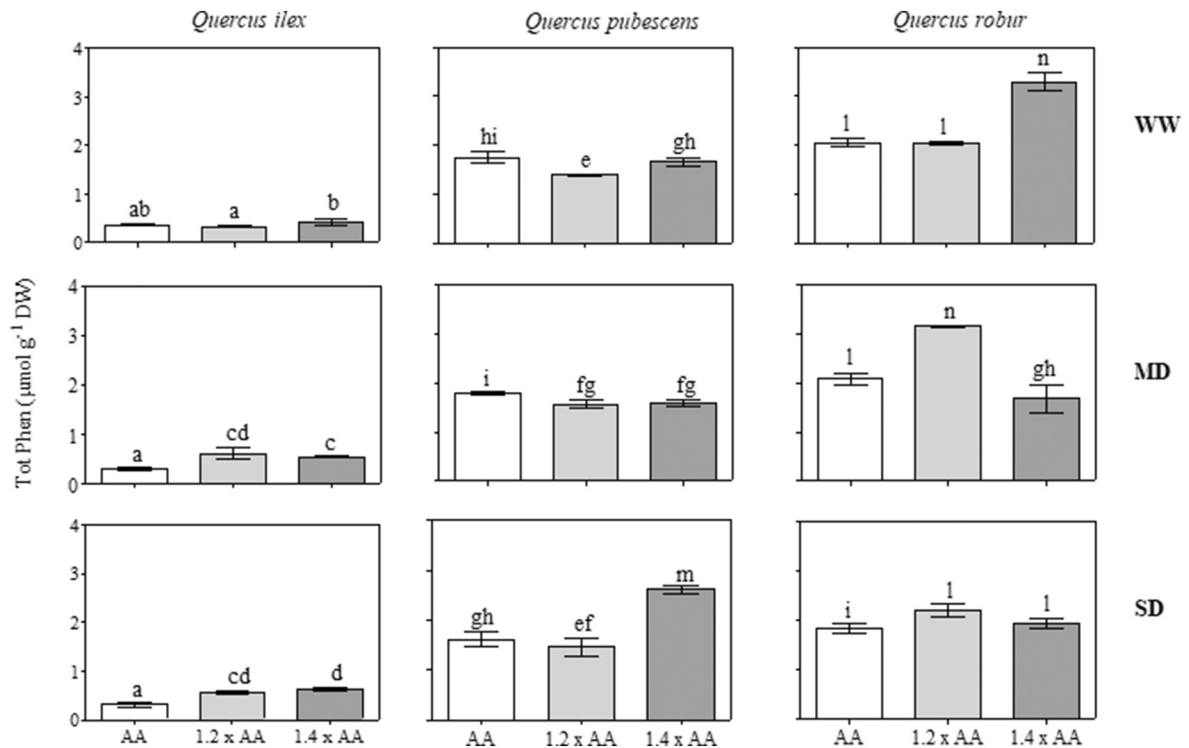
The three-way ANOVA test of total ascorbate (Tot AsA) and total glutathione (Tot Glu) content revealed that the interaction among  $O_3$ , drought and species was not significant (Table 1, Figs. 2S and 3S). The effects of single factors (except “drought” for Tot Glu) were significant. This was also true for the binary interaction “drought  $\times$  species” in the case of Tot AsA.  $O_3$  *per se* had a significant impact on Tot AsA in all the three species under AA conditions. Only moderate  $O_3$  concentrations induced an accumulation of Tot AsA in *Q. ilex* (+21% in comparison to AA; one-way ANOVA with  $O_3$  as factor:  $P \leq 0.01$ ). Drought *per se* had a significant impact on Tot AsA only in *Q. robur*, with significant differences between reduced water availability (one-way ANOVA with drought as

factor:  $P \leq 0.001$ ). *Q. pubescens* displayed Tot AsA values about 2-fold lower than the other species (one-way ANOVA with plant species as factor:  $P \leq 0.01$ ). Ozone *per se* had a slight impact on Tot GSH only in *Q. pubescens* WW plants in comparison to AA ones: moderate  $O_3$  concentrations significantly increased Tot GSH content (+76%). Under AA-WW conditions, *Q. ilex* displayed Tot GSH values about 1.5-fold lower than the other species.

The three-way ANOVA test of Tot Phen and Tot Flav revealed that the interaction among  $O_3$ , drought and species, the effects of each factor (except drought) and their binary interactions were significant (Table 1). Ozone *per se* had a slight impact of Tot Phen only in the deciduous species. Moderate  $O_3$  concentrations negatively altered Tot Phen in *Q. pubescens* WW (−20% compared to AA, Fig. 4). High  $O_3$  concentrations significantly increased Tot Phen in *Q. robur* WW plants (+61 compared to AA). Drought *per se* did not alter Tot Phen in all species (except SD in *Q. robur*). Under AA-WW conditions, *Q. ilex* displayed Tot Phen values about 5-fold lower than the other species, with *Q. robur* showing slightly higher levels than *Q. pubescens*. Under combined conditions ( $O_3$  and drought), Tot Phen content was significantly affected in all species exposed to the higher  $O_3$  concentrations and subjected to reducing watering regimes in comparison to AA. In particular, *Q. ilex* exhibited an  $O_3$ -induced accumulation of Tot Phen under MD and SD conditions,



**Fig. 3.** Total carotenoid (Tot Car) content [ $\mu\text{mol g}^{-1}$  dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O<sub>3</sub> exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O<sub>3</sub> (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean ( $n = 3$ ) ± standard deviation. According to a three-way ANOVA with O<sub>3</sub>, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test,  $P \leq 0.05$ ). Tot Car = neoxanthin + violaxanthin + antheraxanthin + lutein + zeaxanthin +  $\beta$ -carotene.



**Fig. 4.** Total free phenolic (Tot Phen) acid content [ $\mu\text{mol g}^{-1}$  dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O<sub>3</sub> exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O<sub>3</sub> (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean ( $n = 3$ ) ± standard deviation. According to a three-way ANOVA with O<sub>3</sub>, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test,  $P \leq 0.05$ ). Tot Phen = caffeoyl-glucoside + chlorogenic acid + coumaric acid (only in deciduous species) + coumaroylquinic (only in deciduous species) + coumaroyl-glucoside + coumaroyl-glucoside 2 + cryptochlorogenic acid (only in *Q. pubescens*) + gallic acid + galloyl-glucoside + gentisic acid + neochlorogenic acid (only in deciduous species).

without significant differences between the higher O<sub>3</sub> concentrations. Ozone induced a decrease of Tot Phen in *Q. pubescens* MD plants, without significant differences between the higher O<sub>3</sub> concentrations. An opposite trend of Tot Phen was observed in *Q. pubescens* SD and *Q. robur* MD plants in response to increasing O<sub>3</sub> levels: −9 and +61%, +51 and −20% in 1.2 × AA and 1.4 × AA, respectively. Increasing O<sub>3</sub> levels combined with SD induced a slight increase of Tot Phen in *Q. robur*, without significant differences between the higher O<sub>3</sub> concentrations.

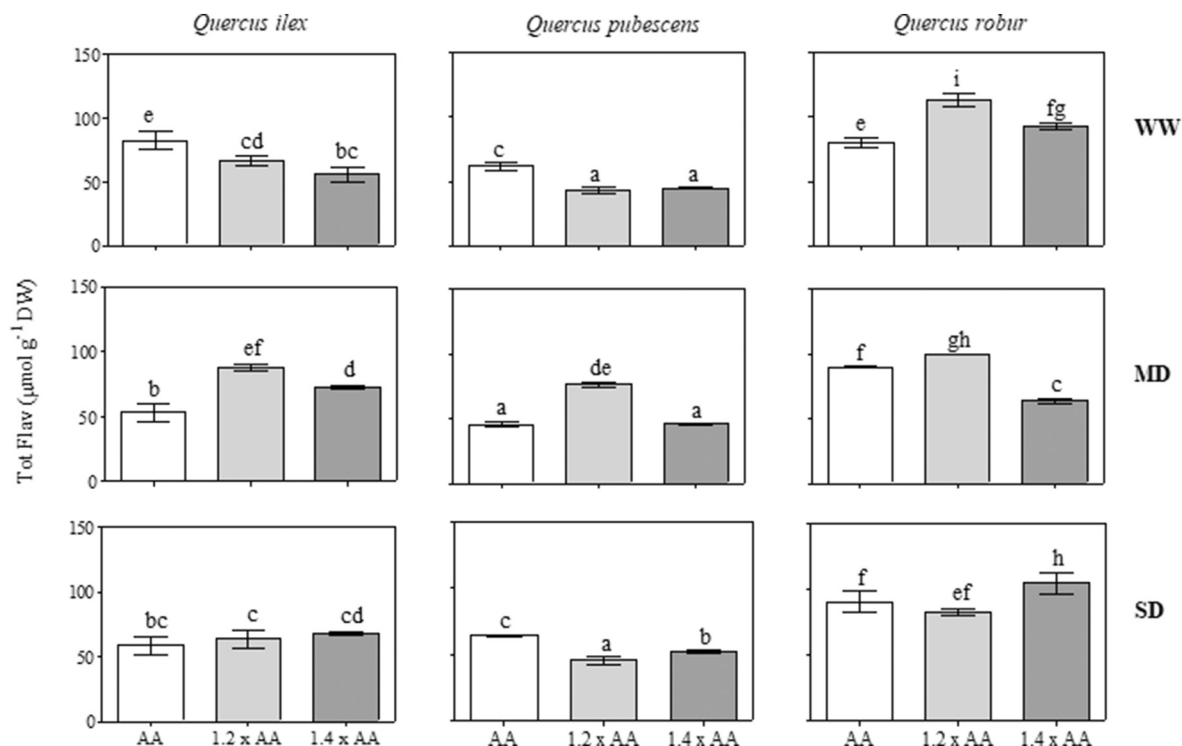
Ozone *per se* significantly affected the content of Tot Flav in all species; in particular, decreased Tot Flav in *Q. ilex* and *Q. pubescens* WW, without significant differences between the higher O<sub>3</sub> concentrations (Fig. 5). An O<sub>3</sub>-induced accumulation of Tot Flav was observed in *Q. robur* WW, with significant differences between O<sub>3</sub> treatments (+43 and +16% in 1.2 × AA and 1.4 × AA, respectively). Drought *per se* significantly influenced Tot Flav in all species (except SD in *Q. pubescens*). Increasing severity of drought induced an evident decrease of Tot Flav in *Q. ilex* under AA, without significant differences between reducing water availability. An MD-induced reduction of Tot Flav was observed in *Q. pubescens* under AA conditions (−27% in comparison to WW). Drought *per se* induced a rise of Tot Flav in *Q. robur* under AA, without significant differences between O<sub>3</sub> levels. Under AA-WW conditions, *Q. pubescens* displayed lower Tot Flav values than the other species. Under combined conditions (O<sub>3</sub> and drought), Tot Flav were significantly affected in all species exposed to increasing O<sub>3</sub> concentrations, independently on the severity of drought (except in *Q. ilex* SD plants). *Q. ilex* MD plants exhibited an O<sub>3</sub>-induced increase of Tot Flav, with significant differences between O<sub>3</sub> levels (+66 and +37% in 1.2 × AA and 1.4 × AA, respectively). Moderate O<sub>3</sub> concentrations markedly increased Tot Flav in *Q. pubescens* MD plants (+69% in comparison to AA ones). Increasing O<sub>3</sub> levels negatively affected Tot Flav in *Q. pubescens* SD plants, with significant differences between O<sub>3</sub> levels (−29 and −19% in 1.2 × AA and 1.4 × AA, respectively). An opposite

trend of Tot Flav was observed in *Q. robur* MD plants exposed to increasing O<sub>3</sub> levels: +11 and −29% in 1.2 × AA and 1.4 × AA, respectively. High O<sub>3</sub> levels slightly altered Tot Flav in *Q. robur* SD plants (+16% compared to AA).

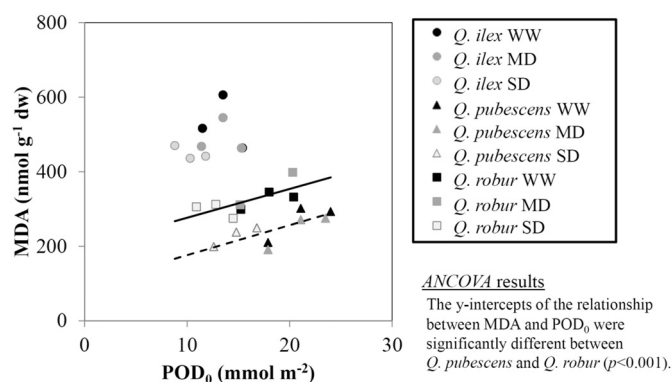
Ozone and drought in combination generally had antagonistic effects on Tot Phen content in all species (Fig. 2G–I). Additive effects were found in *Q. robur* when moderate and high O<sub>3</sub> concentrations were in combination with SD (Fig. 2I). By contrast, O<sub>3</sub> and drought in combination generally had synergistic effects on Tot Flav content in *Q. ilex* (Fig. 2J). Antagonistic effects were found in the deciduous species when moderate and high O<sub>3</sub> concentrations were in combination with MD and SD treatments in *Q. pubescens* and *Q. robur*, respectively (Fig. 2J–L). Weak additive effects were found for Tot Flav in *Q. robur*, when high O<sub>3</sub> concentrations and MD treatments were combined (Fig. 2L).

#### 3.4. Relationship of oxidative metabolism and stomatal ozone uptake (POD)

Higher POD<sub>0</sub> was found in deciduous oaks (*Q. robur*: 10.9 to 20.4 mmol m<sup>−2</sup>; *Q. pubescens*: 12.6 to 24.0 mmol m<sup>−2</sup>) than evergreen oaks (*Q. ilex*: 8.8 to 15.4 mmol m<sup>−2</sup>) (Fig. 6). Soil water deficit limited POD<sub>0</sub> in all three oaks (25 to 30% limitation in SD treatments relative to WW treatments). No significant relationships were found between POD<sub>0</sub> and most of the parameters of oxidative metabolism (*i.e.*, H<sub>2</sub>O<sub>2</sub>, Tot Car, Tot Phen, Tot Flav, AsA, GSH) (*data not shown*). However, we found significant positive relationships between POD<sub>0</sub> and MDA for *Q. pubescens* and *Q. robur*, although such a relationship was not found in *Q. ilex* (Fig. 6). The intercepts of those relationships differed between *Q. pubescens* and *Q. robur* ( $P \leq 0.001$  by ANCOVA), while slopes were comparable ( $\sim 8 \times 10^{-3}$  g DW m<sup>2</sup>).



**Fig. 5.** Total flavonoid (Tot Flav) content [ $\mu\text{mol g}^{-1}$  dry weight (DW)] in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air O<sub>3</sub> exposure [applied for 4.5 months: ambient air (AA), 1.2 × and 1.4 × ambient O<sub>3</sub> (1.2 × AA and 1.4 × AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Data are shown as mean ( $n = 3$ )  $\pm$  standard deviation. According to a three-way ANOVA with O<sub>3</sub>, drought and plant species as factors, different letters show significant differences among bars in the nine graphs (Tukey test,  $P \leq 0.05$ ). Tot Flav = catechin + epicatechin + epigallocatechin + hesperidin + isorhamnetin-glucoside + isorhamnetin-rutinoside + kaempferol (only in *Q. robur*) + kaempferol-glucoside + kaempferol-rutinoside + phloridzin + procyanidin B1 + pelargonidin-rutinoside + quercetin + quercetin arabinoside + quercetin-galactoside + quercetin-galloyl-glucoside + quercetin-glucoside + quercetin-rhamnoside + quercetin-xyloside + rhamnetin-glucoside + rutin.



**Fig. 6.** Relationships between phytotoxic  $O_3$  dose ( $POD_0$ ,  $mmol\ m^{-2}$ ) and malondialdehyde (MDA,  $nmol\ g^{-1}$  dry weight (DW)) in *Quercus ilex*, *Q. pubescens* and *Q. robur* leaves under free air  $O_3$  exposure [applied for 4.5 months: ambient air (AA),  $1.2 \times$  and  $1.4 \times$  ambient  $O_3$  ( $1.2 \times$  AA and  $1.4 \times$  AA)] and subjected to different watering regimes [well-watered (WW), moderate drought (MD) and severe drought (SD)]. Significant relationships were found in *Q. pubescens* (dotted line,  $y = 8.05x + 95.74$ ,  $R^2 = 0.58$ ,  $P < 0.05$ ) and *Q. robur* (black line,  $y = 7.72x + 199.49$ ,  $R^2 = 0.50$ ,  $P < 0.05$ ), while no significant regression was found in *Q. ilex* ( $y = 6.21x + 413.15$ ,  $R^2 = 0.06$ ,  $P = 0.52$ ). The y-intercepts were significantly different between *Q. pubescens* and *Q. robur* by an ANCOVA test ( $P < 0.001$ ). ns denotes not significant.

#### 4. Discussion

Plants are frequently exposed to adverse environmental conditions such as drought and  $O_3$ . Under these conditions, plants can survive thanks to the ability to adjust their metabolism to cope with these stressors (Noctor et al., 2018). Much progress has been made in understanding the dual role of ROS in plant biology. ROS are required for many important signalling reactions, but are also toxic by-products of aerobic metabolism. This dual role is mainly dependent on (i) their concentration, (ii) site and duration of their action, (iii) occurrence of previous stress events and (iv) concurrence of other constrained conditions (Noctor et al., 2018). At high concentrations, ROS pose a significant threat that may eventually lead to programmed cell death (PCD). At low doses, ROS are employed as signals that mediate at least part of the responses towards stress. ROS production is a common feature under abiotic stress conditions (Foyer and Noctor, 2011).

ROS accumulation can also be due to  $O_3$  degradation into the leaf tissues (Czarnocka and Karpiński, 2018). Drought response mostly uses ROS as internally produced signalling molecules, although severe drought may lead to photo-oxidative stress (Reddy et al., 2004). Recent evidence shows that when subjected to a combination of multiple stresses, plants respond differently relative to when they experience only a single type of stress (Cotrozzi et al., 2017b). Therefore, the first question we wanted to address for the three species investigated in the present study was “How much ROS are induced by realistic  $O_3$  and water stress levels”? The impact of  $O_3$  *per se* on ROS production appeared to be species-specific in view of the accumulation of  $H_2O_2$  observed only in *Q. ilex*, where however the basal levels of this ROS were lower compared to the deciduous species, likely due to a balancing relation with other ROS (e.g., anion superoxide, hydroxyl radical; Foyer and Noctor, 2011). The induction of an oxidative burst by the two higher  $O_3$  concentrations, however, was not associated with membrane injury (as demonstrated by the unchanged MDA by-product values). This result suggests that an activation of an efficient free radical scavenging system minimized the adverse effects of a general peroxidation, thus contributing to (i) the maintenance of membrane structure and integrity and (ii) the delay of leaf senescence (Miller et al., 1999).

Peroxidation control and cell membrane stability under increased ROS conditions are usually characteristic of  $O_3$ -tolerant plants, which are able to cope with ROS by the activation of enzymatic and non-enzymatic antioxidant compounds (Gill and Tuteja, 2010). In contrast, the deciduous species increased MDA under  $O_3$  as  $H_2O_2$  likely reacted

with some cell wall and plasma membrane components, which resulted in lipid peroxidation (Czarnocka and Karpiński, 2018). Drought *per se* also induced a similar  $H_2O_2$  accumulation in *Q. ilex*, but again, the maintenance of membrane functionality (i.e., unchanged MDA by-product values) suggests that a tight control of ROS production occurred. In particular,  $H_2O_2$  may be involved in the integration of cellular processes and in the adaptation to environmental stimuli (Dizengremel et al., 2013). The reducing water availability had a strong impact on the deciduous species. A marked over-production of  $H_2O_2$  was observed in *Q. pubescens* SD and *Q. robur* MD plants and it was associated with membrane denaturation. This result suggests that oxidative damage occurred, probably due to an inadequate response of the antioxidative systems (Czarnocka and Karpiński, 2018).

In light of the above, the second question was “Which antioxidant mechanisms are activated in response to individual stresses and to the combination of the stressors at different intensities”? It is known that leaf biochemical traits (e.g., Car, AsA, GSH, Phen, Flav) are crucial for avoiding and preventing oxidative damage during stress conditions (Sharma et al., 2012). Our results suggest that the oxidative damage induced by the higher  $O_3$  concentrations and the reduced water availability (single or in combination) only slightly modified the pool (and the reduction level) of the metabolites involved in the Halliwell-Asada cycle. Only  $O_3$  *per se* increased the total abundance of intracellular AsA in all the three species under AA conditions, confirming that AsA represents the first line of defence against  $O_3$ -oxidative load (Conklin and Barth 2004). Particularly, a special role could be attributed to the apoplastic ascorbate. This fraction could contribute to differences in  $O_3$  tolerance for *Quercus* species as shown for other species (Burkey et al., 2000; Feng et al., 2010). However, AsA did not seem to be sufficient to mitigate the negative effects of  $O_3$  in terms of ROS production (in *Q. ilex*) and membrane denaturation (in *Q. pubescens* and *Q. robur*), suggesting that it may be more important in terms of regulation than in redox homeostasis (Foyer and Noctor, 2011). This is probably because AsA is a cofactor of several plant-specific enzymes that are involved in important pathways leading to the biosynthesis of (i) cell wall hydroxyproline-rich proteins, (ii) defence-related secondary metabolites and (iii) plant hormones (Gest et al., 2013). Our results indicate that the phenylpropanoid pathway (including non-volatile isoprenoids such as carotenoids) was very responsive in stressed plants.

It is known that Car are liposoluble antioxidants that play several functions in plant metabolism including oxidative stress tolerance (Havaux et al., 2005). They serve an important photoprotective role by dissipating excess excitation energy as heat or by scavenging ROS and suppressing lipid peroxidation (Gill and Tuteja, 2010). Consequently, Car can transiently complement the action of the primary antioxidants (i.e., AsA and enzymatic antioxidant compounds, Brunetti et al., 2015). Secondary metabolites (such as phenols and flavonoids) are well suited to constitute a “secondary” antioxidant system with a central role in plant defence against severe constraints by avoiding the generation of ROS and by quenching ROS once they are formed (Brunetti et al., 2015). For all the three species tested in this work, it is possible to conclude that distinct phenylpropanoid pathways were activated in response to  $O_3$  and drought, when applied singularly. Ozone and drought in combination generally had antagonistic effects on most biochemical traits. This was most evident for Tot Car in *Q. ilex* (except when both stressors were of severe intensity) and *Q. robur*, and for Tot Phen in *Q. ilex* and *Q. pubescens*, as well as for ROS production, in which the accumulation of  $H_2O_2$  in *Q. ilex* and *Q. pubescens* under  $1.4 \times$  AA-SD conditions was not as severe as expected. However, Tot Flav (the most representative class of phenylpropanoid compounds) was affected by both stressors in *Q. ilex* (at different intensities) and in *Q. pubescens* and *Q. robur* under both  $1.4 \times$  AA-SD and  $1.2 \times$  AA-MD conditions, by exhibiting relatively strong and synergistic effects relative to their combined impact. It is possible to hypothesize that the strong decrease of Tot Phen could be related to the consumption of these compounds by the cell to counteract the accumulation of  $H_2O_2$ , thus representing an



important defence mechanism against the increased oxidative metabolism induced by reduced water availability. This result confirms that Tot Flav can be considered as a robust biochemical trait to improve the adaptability of plants to harsh environments. However, this mechanism appeared to be species-specific and depended on the so-called “metabolic plasticity” (Logemann et al., 2000).

In light of the above, the third question was “Are metabolic responses markedly species-specific”? In *Q. ilex*,  $O_3$  *per se* induced an increase of Tot Car that inhibited and/or prevented the peroxidation action of free radicals, as confirmed by unchanged MDA by-product values. The concomitant decrease of Tot Flav could be related to the consumption of these compounds by the cell to counteract the accumulation of  $H_2O_2$ , thus representing an important defence mechanism against the increased oxidative metabolism induced by  $O_3$  (Pellegrini et al., 2018). The unchanged Tot Phen values confirmed that *Q. ilex* can be considered  $O_3$ -tolerant according to the evidence that the biosynthesis of phenylpropanoids increases more in stress-sensitive than in tolerant species (Fini et al., 2012; Cotrozzi et al., 2018b). Drought *per se* did not give rise to the same effects induced by  $O_3$  confirming that the biochemical features found in *Q. ilex* are enough to explain its superior ability to counteract unfavorable environmental conditions, also in terms of the reducing water availability. In *Q. pubescens*, high  $O_3$  concentrations *per se* induced a concomitant reduction of Tot Car and Tot Flav suggesting that these antioxidants could be consumed by the cell to counteract the possible ROS generation due to increased oxidative metabolism and cellular damages. It is known that Car and Flav are involved in non-photochemical quenching mechanisms, thus reducing the risk of photo-oxidative stress (Niinemets et al., 2003). Based on relative physical-chemical features and intra-cellular distribution, they may serve distinct and complementary functions (Close and Beadle, 2003). Consequently, the utilization of these compounds could improve the tolerance of *Q. pubescens* to  $O_3$ , as confirmed by the unchanged Tot Phen values. For this species, moderate drought *per se* did not give rise to the same effects induced by  $O_3$ . This result confirms that the biochemical features found in *Q. pubescens* increased the ability of cells to scavenge stress-derived ROS, but they counteracted the negative impact of both stressors (at different intensities) less efficiently than in the sympatric *Q. ilex*. In *Q. robur*, high  $O_3$  concentrations *per se* induced a partial rearrangement of the phenylpropanoid pathways with different functions in order to alleviate the excess of excitation pressure and to provide antioxidative protection to chloroplasts.

Phenylpropanoid pathways contribute to all aspects of plant responses towards biotic and abiotic stimuli (Vogt, 2010). Generally, the increase in phenylpropanoid concentration can be considered a repair process that can equip stressed plants with an additional antioxidant system capable of avoiding and scavenging ROS (Cotrozzi et al., 2018b). It is possible to conclude that the reduction of Tot Car and the concomitant increase of Tot Flav were not enough to counteract and/or reduce the photo-oxidative stress induced by high  $O_3$  concentrations. The concomitant induction of Tot Phen would indicate a better capacity to regulate the level of ROS, and hence the cellular redox state. However, these additional antioxidative mechanisms were not able to protect and/or repair the cell structure and to prevent the occurrence of the oxidative load (as confirmed by the increase of MDA by-products values). Severe drought *per se* did not give rise to the same effects induced by high  $O_3$  concentrations confirming that *Q. robur* is least adapted to unfavorable environmental conditions because of a lower degree of tolerance compared with the other two species. Even though no clear relationship between the activation of phenylpropanoids and stress tolerance has been established, it is known that the biosynthesis of these secondary metabolites increases more in stress-sensitive than in tolerant species (Fini et al., 2012). The variation in  $O_3$ -sensitivity among the three species tested in this work can be ascribed not only to the ability of cells to scavenge  $O_3$ -derived ROS and to raise detoxifying barriers, but also to the stomatal  $O_3$  uptake. According to Reich (1987), cell fate in an  $O_3$ -polluted environment depends on exposure, uptake and

biological responses, so a powerful tool to estimate plant susceptibility to  $O_3$  should take into account both cellular biochemical defenses and  $O_3$  flux through stomata.

In light of the above, the fourth question was “Are oxidative metabolism and stomatal uptake of  $O_3$  correlated”? Treutler (2005) reported that the antioxidant compounds can be divided into two groups: “preformed” and “induced” compounds, the latter being synthesized by plants in response to physical injury, infection or stress. Concerning our results, it is possible to conclude that no significant relationships were found between most of the parameters of antioxidant metabolism and  $POD_0$  suggesting that an unbalance between repair (*i.e.*, the capacity to activate detoxifying systems) and avoidance strategies (*i.e.*, the ability of leaves to partially close stomata to exclude  $O_3$  from leaf intercellular space) occurred. Although recent studies documented that the  $POD$  approach can be considered the best metric to assess  $O_3$  effects on (i) plant productivity (*i.e.*, biomass and yield losses, leaf mass per area *etc.*), (ii) photosynthetic performance and (iii) visible foliar injury (Gao et al., 2017; Hoshika et al., 2018), the internal mechanism of  $O_3$  sensitivity is controversial. Some studies found that  $O_3$  sensitivity was associated with high stomatal conductance (*g*, Wittig et al., 2007; Cotrozzi et al., 2016; Yang et al., 2016), but others argued that it depended on antioxidant levels (Nali et al., 2004; Dai et al., 2017). In addition, a wide range of different biochemical, structural and physiological leaf traits can play a pivotal role in determining  $O_3$  response. The relative contributions of these different traits in controlling the inter-specific variation in  $O_3$  sensitivity among a wide range of species remain elusive (Li et al., 2017). A significant and positive relationship between MDA and  $POD_0$  was found in *Q. pubescens* and *Q. robur*, confirming that the greater stomatal  $O_3$  flux in these deciduous oak species (according to biomass results from Hoshika et al., 2018) resulted in pronounced membrane denaturation.

It is possible to conclude that the  $POD$  approach can be used for assessing accelerated leaf senescence in deciduous oak species. In fact, no similar relationship was found in *Q. ilex* that under AA-WW conditions displayed not only lower amounts of AsA, GSH, Tot Car and Tot Phen than the other species, but also the lowest values of  $POD_0$  suggesting that intrinsic physiological and biochemical mechanisms can contribute significantly to the stress tolerance. To conclude,  $O_3$  and drought had antagonistic effects on most biochemical traits which depended on plant species. In particular, we untangled the species-specific biochemical adjustments that may reduce the impact of  $O_3$  when combined with the effect of drought.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.07.413>.

## References

- Alonso, R., Elvira, S., Gonzalez-Fernandez, I., Calvete, H., Garcia-Gomez, H., Bermejo, V., 2014. Drought stress does not protect *Quercus ilex* L. from ozone effects: results from a comparative study of two subspecies differing in ozone sensitivity. *Plant Biol.* 16, 375–384.
- Assumpção, C.F., Herms, V.S., Pagno, C., Castagna, A., Mannucci, A., Sgherri, C., et al., 2018. Phenolic enrichment in apple skin following post-harvest fruit UV-B treatment. *Post-harvest Biol. Technol.* 138, 37–45.
- Avery, R.J., 2006. Reactivity-based VOC control for solvent products: more efficient ozone reduction strategies. *Environ. Sci. Technol.* 40, 4845–4850.
- Bansal, S., Hallsby, G., Löfvenius, M.O., Nilsson, M.-C., 2013. Synergistic, additive and antagonistic impacts of drought and herbivory on *Pinus sylvestris*: leaf, tissue and whole-plant responses and recovery. *Tree Physiol.* 33, 451–463.

- Bohler, S., Cuypers, A., Vangronsveld, J., 2015. Interactive effects between ozone and drought: sorrow or joy? In: Mahalingam, R. (Ed.), Combined Stresses in Plants - Physiological, Molecular, and Biochemical Aspects. Springer International Publishing, Switzerland, pp. 147–157.
- Brunetti, C., Guidi, L., Sebastiani, F., Tattini, M., 2015. Isoprenoids and phenylpropanoids are key components of the antioxidant defense system of plants facing severe excess light stress. *Environ. Exp. Bot.* 119, 54–62.
- Burkey, K.O., Wei, C., Eason, G., Ghosh, P., Fenner, G.P., 2000. Antioxidant metabolite levels in ozone-sensitive and tolerant genotypes of snap bean. *Physiol. Plant.* 110, 195–200.
- Bussotti, F., Bettini, D., Grossoni, P., Mansueto, S., Nibbi, R., Soda, C., Tani, C., 2002. Structural and functional traits of *Quercus ilex* in response to water availability. *Environ. Exp. Bot.* 47, 11–23.
- Butkovic, V., Cvitas, T., Klasing, L., 1990. Photochemical ozone in the Mediterranean. *Sci. Total Environ.* 99, 145–151.
- Chaves, M.M., Maroco, J.P., Pereira, J.S., 2003. Understanding plant responses to drought: from genes to the whole plant. *Funct. Plant Biol.* 30, 239–264.
- Close, D.C., Beadle, C.L., 2003. Alternate energy dissipation? Phenolic metabolites and the xanthophyll cycle. *J. Plant Physiol.* 160, 431–434.
- CLRTAP, 2017. Mapping critical levels for vegetation. Chapter III of manual on methodologies and criteria for modelling and mapping critical loads and levels and air pollution effects, risks and trends. UNECE Convention on Long-range Transboundary Air Pollution. [www.icpmapping.org](http://www.icpmapping.org). Accessed date: 28 February 2018.
- Conklin, P.L., Barth, C., 2004. Ascorbic acid, a familiar small molecule intertwined in the response of plants to ozone, pathogens, and the onset of senescence. *Plant Cell Environ.* 27, 959–970.
- Cotrozzi, L., Remorini, D., Pellegrini, E., Landi, M., Massai, R., Nali, C., Guidi, L., Lorenzini, G., 2016. Variations in physiological and biochemical traits of oak seedlings grown under drought and ozone stress. *Physiol. Plant.* 157, 69–84.
- Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Lorenzini, G., Massai, R., Nali, C., Landi, M., 2017a. Cross-talk between physiological and metabolic adjustments adopted by *Quercus cerris* to mitigate the effects of severe drought and realistic future ozone concentrations. *Forests* 8, 148. <https://doi.org/10.3390/f8050148>.
- Cotrozzi, L., Pellegrini, E., Guidi, L., Landi, M., Lorenzini, G., Massai, R., Remorini, D., Tonelli, M., Trivellini, A., Vernieri, P., Nali, C., 2017b. Losing the warning signal: drought compromises the cross-talk of signaling molecules in *Quercus ilex* exposed to ozone. *Front. Plant Sci.* 8, 1020. <https://doi.org/10.3389/fpls.2017.01020>.
- Cotrozzi, L., Remorini, D., Pellegrini, E., Guidi, L., Nali, C., Lorenzini, G., Massai, R., Landi, M., 2018a. Living in a Mediterranean city in 2050: broadleaf or evergreen “citizens”? *Environ. Sci. Pollut. Res.* 25, 8161–8173.
- Cotrozzi, L., Campanella, A., Pellegrini, E., Lorenzini, G., Nali, C., Paoletti, E., 2018b. Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, *Fraxinus excelsior*. *Environ. Sci. Pollut. Res.* 25, 8137–8147.
- Curtu, A.L., Șofletea, N., Toader, A.V., Enescu, C.M., 2011. Leaf morphological and genetic differentiation between *Quercus robur* L. and its closest relative, the drought tolerant *Q. pedunculiflora* K. Kock. *Ann. For. Sci.* 68, 1163–1172.
- Czarnocka, W., Karpiński, S., 2018. Friend or foe? Reactive oxygen species production, scavenging and signaling in plant response to environmental stresses. *Free Radic. Biol. Med.* <https://doi.org/10.1016/j.freeradbiomed.2018.01.011>.
- Dai, L., Li, P., Shang, B., Liu, S., Yang, A., Wang, Y., Feng, Z., 2017. Differential responses of peach (*Prunus persica*) seedlings to elevated ozone are related with leaf mass per area, antioxidant enzymes activity rather than stomatal conductance. *Environ. Pollut.* 227, 380–388.
- Davey, M.W., Dekempener, E., Keulemans, J., 2003. Rocket-powered high-performance liquid chromatographic analysis of plant ascorbate and glutathione. *Anal. Biochem.* 316, 74–81.
- Dizengremel, P., Jolivet, Y., Tuzet, A., Ranieri, A., Le Thiec, D., 2013. Integrative leaf-level phytotoxic ozone dose assessment for forest risk modelling, in: Matissek, R., Clarke, N., Cudlin, P., de Carvalho, M.H.C., 2008. Drought stress and reactive oxygen species. *Plant Signal. Behav.* 3, 156–165.
- EEA, 2017. Air quality in Europe – 2017 report. EEA Report No17/20217. European Environment Agency (<https://www.eea.europa.eu/publications/air-quality-in-europe-2017>).
- Feng, Z., Pang, J., Nouchi, I., Kobayashi, K., Yamakawa, T., Zhu, J., 2010. Apoplastic ascorbate contributes to the differential ozone sensitivity in two varieties of winter wheat under fully open-air field conditions. *Environ. Pollut.* 158, 3539–3545.
- Fini, A., Guidi, L., Ferrini, F., Brunetti, C., Di Fernando, M., Biricolti, S., Pollastri, S., Calamai, L., Tattini, M., 2012. Drought stress has contrasting effects on antioxidant enzymes activity and phenylpropanoid biosynthesis in *Fraxinus ornus* leaves: an excess light stress affair? *J. Plant Physiol.* 169, 929–939.
- Foyer, C.H., Noctor, G., 2011. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* 155, 2–18.
- Gao, F., Catalayud, V., Paoletti, E., Hoshika, Y., Feng, Z., 2017. Water stress mitigates the negative effects of ozone on photosynthesis and biomass in poplar plants. *Environ. Pollut.* 230, 268–279.
- Gest, N., Gautier, H., Stevens, R., 2013. Ascorbate as seen through plant evolution: the rise of a successful molecule? *J. Exp. Bot.* 64, 33–53.
- Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* 48, 909–930.
- Guidi, L., Remorini, D., Cotrozzi, L., Giordani, T., Lorenzini, G., Massai, R., Nali, C., Natali, L., Pellegrini, E., Trivellini, A., Vangelisti, A., Vernieri, P., Landi, M., 2017. The harsh life of an urban tree: the effect of a single pulse of ozone in salt-stressed *Quercus ilex* saplings. *Tree Physiol.* 37, 246–260.
- Havaux, M., Eymery, F., Porfirova, S., Rey, P., Dörmann, P., 2005. Vitamin E protects against photoinhibition and photooxidative stress in *Arabidopsis thaliana*. *Plant Cell* 17, 3451–3469.
- Hoshika, Y., Moura, B., Paoletti, E., 2018. Ozone risk assessment in three oak species as affected by soil water availability. *Environ. Sci. Pollut. Res.* 25, 8125–8136.
- Jolivet, Y., Bagard, M., Cabané, M., Vaultier, M.N., Gandin, A., Afif, D., Dizengremel, P., Le Thiec, D., 2016. Deciphering the ozone-induced changes in cellular processes: a prerequisite for ozone risk assessment at the tree and forest levels. *Ann. For. Sci.* 73, 923–943.
- Li, P., Feng, Z., Catalayud, V., Yuan, X., Xu, Y., Paoletti, E., 2017. A meta-analysis on growth, physiological, and biochemical responses of woody species to ground-level ozone highlights the role of plant functional types. *Plant Cell Environ.* 40, 2369–2380.
- Logemann, E., Tavernaro, A., Schulz, W., Somssich, I.E., Hahlbrock, K., 2000. UV light selectively coincides supply pathways from primary metabolism and flavonoid secondary product formation in parsley. *Proc. Natl. Acad. Sci.* 97, 1903–1907.
- Miller, J.D., Arteca, R.N., Pell, E.J., 1999. Senescence-associated gene expression during ozone-induced leaf senescence in *Arabidopsis*. *Plant Physiol.* 120, 1015–1023.
- Nali, C., Paoletti, E., Marabottini, R., Della Rocca, G., Lorenzini, G., Paolacci, A.R., Ciaffai, M., Badiani, M., 2004. Ecophysiological and biochemical strategies of response to ozone in Mediterranean evergreen broadleaf species. *Atmos. Environ.* 38, 2247–2257.
- Niinemets, Ü., Kollist, H., García-Plazaola, J.I., Hernández, A., Becerril, J.M., 2003. Do the capacity and kinetics for modification of xanthophyll cycle pool size depend on growth irradiance in temperate trees? *Plant Cell Environ.* 26, 1787–1802.
- Noctor, G., Reichheld, J.P., Foyer, C.H., 2018. ROS-related redox regulation and signaling in plants. *Semin. Cell Dev. Biol.* 80, 3–12.
- Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D., Badea, O., 2017. A new-generation 3D ozone Face (Free Air Controlled Exposure). *Sci. Total Environ.* 575, 1407–1414.
- Pellegrini, E., Campanella, A., Cotrozzi, L., Tonelli, M., Nali, C., Lorenzini, G., 2018. What about the detoxification mechanisms underlying ozone sensitivity in *Liriodendron tulipifera*? *Environ. Sci. Pollut. Res.* 25, 8148–8160.
- Pollastri, M., Desotgiu, R., Camin, F., Ziller, L., Marzuoli, R., Gerosa, G., Bussotti, F., 2013. Intra-annual pattern of photosynthesis, growth and stable isotope partitioning in a poplar clone subjected to ozone and water stress. *Water Air Soil Pollut.* 224, 1761–1772.
- Reddy, A.R., Chaitanya, K.V., Vivekanandan, M., 2004. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. *J. Plant Physiol.* 161, 1189–1202.
- Reich, P.B., 1987. Quantifying plant response to ozone: a unifying theory. *Tree Physiol.* 3, 63–91.
- Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanisms in plants under stressful conditions. *J. Bot.* 217037. <https://doi.org/10.1155/2012/217037>.
- Treutter, D., 2005. Significance of flavonoids in plant resistance and enhancement of their biosynthesis. *Plant Biol.* 7, 581–591.
- Vogt, T., 2010. Phenylpropanoid biosynthesis. *Mol. Plant* 3, 2–20.
- Vranckx, G., Jacquemyn, H., Mergey, J., Cox, K., Janssens, P., Gielem, B.A.S., Muys, B., Honnay, O., 2014. The effect of drought stress on heterozygosity-fitness correlations in pedunculated oak (*Quercus robur*). *Ann. Bot.* 113, 1057–1069.
- Wittig, V.E., Ainsworth, E.A., Long, S.P., 2007. To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review of the last 3 decades of experiments. *Plant Cell Environ.* 30, 1150–1162.
- Yang, N., Wang, X., Cotrozzi, L., Chen, Y., Zheng, F., 2016. Ozone effects on photosynthesis of ornamental species suitable for urban green spaces of China. *Urban For. Urban Green.* 20, 437–447.
- Yuan, X., Calatayud, V., Gao, F., Fares, S., Paoletti, E., Tian, Y., Feng, Z., 2016. Interaction of drought and ozone exposure on isoprene emission from extensively cultivated poplar. *Plant Cell Environ.* 39, 2276–2287.